Confidential

THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of : Lal et al.

Application No: 10/611,539

Filed : July 1, 2003

For : Inhibitors Of Cyclin-Dependent Kinases And Their Use

Commissioner of Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

DECLARATION UNDER 37 C. F. R. § 1.132

I, Kalpana S. Joshi, declare as follows:

1. I am citizen of India.
2. My educational background is as follows:
   1981: **M.Sc.** Molecular Biology, Pune University, Pune India.
   1987: **Ph.D.** Cancer Biology, Cancer Research Institute, Tata Memorial Centre, Mumbai, India
   1987-1992: Postdoctoral research, University Iowa, Iowa City, USA
3. I am currently Director, Oncology at Nicholas Piramal India Ltd.
   From 1997-2005 I had been Senior Principal Research Scientist, Oncology, Nicholas Piramal India Limited, India; and from 1992 to 1997 I had been Group Leader-Oncology & Inflammation, Drug Discovery, Hoechst Marion Roussel Research Center, Mumbai, India.

4. I am named author on 17 journal publications, 2 patents and 30 published abstracts.

5. I am a co-inventor of U.S. Application No. 10/611,539, filed July 1, 2003.

6. I have reviewed the specification and claims of the present application. The present application is directed to compounds for the inhibition of cyclin-dependent kinases and their use.

7. I have also reviewed the Office Action dated April 19, 2006. I understand that the U.S. Patent and Trademark Office examiner has rejected claims 12-13 and 25-26 for lacking enablement for all cancers. The examiner has stated that the specification does not enable any person skilled in the art to use the invention commensurate in scope with the claims. I understand that at page(s) 5-6 of the office action under the heading "*The State Of The Prior Art And The Predictability Or Lack Thereof In The Art*", the examiner has contended that: in the absence of a showing of correlation between all cancers claimed as capable of treatment by inhibiting cyclin-dependent kinases (Cdks), one skilled in the art is unable to predict possible results from the administration of the compound of formula 1c due to unpredictability of the role of inhibiting Cdks. As a person of skill in the art, I do not agree with the examiner's contention that there is no showing of correlation between cyclin dependent kinases and the development of cancer. I am providing herein below the evidentiary support to elucidate the role of cyclin dependent kinases in the development of cancer. I am also providing herein below data to support my contention that the compounds of formula 1c, which are Cdk inhibitors, can inhibit proliferation of many cancer cells.

8. **Correlation between Cdks and the development of cancer:**

Deregulation of cell cycle control mechanisms is an obligatory step in tumorigenesis. Myriad individual genetic events lead to circumvention of checkpoints that restrain the activity of cyclin/cyclin-dependent kinase (Cdk) complexes that are responsible for managing cell cycle transitions. Indeed, it is now wildly accepted that deregulation of retinoblastoma protein (pRb) pathway

occurs in virtually all-human tumors. It is also known to persons skilled in the art that most abnormalities occur due to hyperphosphorylation of the tumor suppressor gene Rb by the key regulators of the cell cycle, the cyclin-dependent kinases (Cdks). The tumor suppressor gene Rb is an important component in the G1/S transition. Therefore, modulation of Cdks is an important use for the therapy and prevention of human neoplasms. Our objective was to discover and develop an anti-proliferative novel compound with specific inhibition of Cdk enzymes. Recent discoveries have brought several cell cycle regulators into sharp focus as factors involved in human cancer and among these Cdks appear to be most promising ones for pharmacological intervention. Thus clearly indicating that Cdk inhibitors can inhibit proliferation of many cancer cells and this property is not restricted to any particular cancers. Several small-molecule Cdk modulators are being discovered and tested in the clinic.

An increasing body of references has also shown a link between cancer and Cdk related malfunction. Therefore, the cyclin dependent kinases, are suitable as a target of anti-cancer agents. These kinases also become a target in developing small molecule inhibitors for suppression or treatment of cancer. E. Sausville et al in "The Oncologist 2001; 6:517-537" provides report for oncogenic alteration of cyclins, cyclin dependent kinases and cyclin dependent kinase inhibitors in various human cancers. This reference also emphasises that: "Inhibition of Cdks is particularly attractive from the perspective of anti-cancer drug design given their pivotal role in the cell cycle". E. Sausville in an article entitled "Cyclin dependent kinases: Novel targets For Cancer Treatment ", Pages 9-21 teaches that cancer is frequently accompanied by loss of regulation of cell-cycle progression through altered expression of Cdk components.

The two references: Davies (Pharmacology & Therapeutics, 2002, 125-133) and Toogood (Medicinal Research Reviews, 2001, 487-498) cited by the examiner also ascertain the nexus between Cdks and cancer. For instance, Davies emphasises that strong genetic link between Cdks and the molecular pathology

of cancer has provided the rationale for developing small-molecule inhibitors of Cdks. The Toogood reference briefly reviews the use of a class of pyrido[2,3-d]pyrimidines compounds as Cdk inhibitors. Although, both these reference do not provide actual data for treatment of cancer, they ascertain that the inhibitors of cyclin-dependent kinases possess therapeutic utility against a wide variety of proliferative diseases, especially cancer.

9. Data for cyclin dependent kinase inhibitors as anticancer agents reported in the prior art:

The finding that a variety of genetic and epigenetic events cause universal overactivity of the cell cycle Cdks in human cancer, and their inhibition can lead to both cell cycle arrest and apoptosis led to the development of Cdk inhibitors as anti-cancer agents. A number of compounds having potentially useful Cdkinhibitory properties have been identified and reported in the literature.

Flavopiridol is the first potent inhibitor of cyclin-dependent kinases (cdks) to reach clinical trial. It has been reported that flavopiridol induces cell cycle arrest and tumor growth inhibition in the majority of solid tumor cell lines and xenografts. For instance: Parker BW et al in Blood 1998 15 : 91(2): 458-65, reported anti-proliferative effects *in vitro* of flavopiridol. This reference reported the effect of flavopiridol on cell cycle progression in different cell types including SUDHL4, SUDHL6 (B-cell lines), Jurkat, and MOLT4 (T-cell lines), and HL60 (myeloid) and observed that the cell lines displayed notable sensitivity to flavopiridol-induced apoptosis.

E. Sausville et al. in "The Oncologist 2001; 6:517-537" refers to a number of scientific articles wherein the "*In vitro* cell culture and *in vivo* animal xenograft studies revealed that Flavopiridol causes significant inhibition of various tumor cell lines including breast, lung, colon COL0205, prostate DU145, lymphoma HL60 and SUDHL4 and head and neck HN-12.

Also E. Sausville's publication: Pharmacol.Ther. 82, 285-292, 1999) indicates clearly that inhibitors of Cdks are active against many cancers.

L Meijer's publication (Pharmacol.Ther. 82, 279-282, 1999) also teaches potential applications of Cdk inhibitors.

10. Evidence of enablement for the use of compounds of formula 1c in the treatment of various cancers :

I would like to point out here that the cited reference namely: Toogood (Medicinal Research Reviews, 2001, 487-498) teaches compounds, which are very specific inhibitors of Cdk4-D1 (viz. Compound #56 for which the biological activity is given). Further he has shown that these are specific to Rb (retinoblastoma) + ve cells as it is a substrate of Cdk4 and not active on Rb –ve cells. It produces a distinct G1 block, which is consistent with specific Cdk4-D1 inhibition. Another cited reference namely Davies et al. (Pharmacology & Therapeutics, 2002, 125-133) discusses the role of Cdk2 specific inhibitors in cell cycle. The compounds represented in this paper are once again specific for Cdk2 and do not show activity on Cdk4 or Cdk1.

On the other hand the compounds of the present invention, besides inhibiting the Cdk4, also inhibits other Cdks as shown in kinase specificity **Table 1** for hydrochloride salt of (+)-*trans*-2-(2-chloro-phenyl)-5,7-dihydroxy-8-(2-hydroxy-methyl-1-methyl-pyrrolidin-3-yl)-chromen-4-one, a representative compound in the series. It also has potent antiproliferative activity on both Rb + and Rb –ve (SaOS-2) cells (**Table 2**) and shows G1 and G2 arrest in asynchronous cell population which is consistent with Cdk4/Cdk1 inhibition. Besides the ability to inhibit Cdk4-D1, representative compound also potently inhibit Cdk1-B and Cdk9-T1 as a result the activity of it is not limited to Rb +ve cancers.

Table 1

***In vitro* kinase assay in the presence of representative compound**

Sr. no.	Kinase	Representative Compound IC_{50} (μM)
1	Cdk4-D1	0.063 ± 0.018
2	Cdk2-E	2.540 ± 0.409
3	Cdk1-B	0.079
4	Cdk9-T1	0.02

To support the contention that the compounds of the formula Ic of the instant invention are useful for the treatment of variety of cancers, I am providing in the following Table 2 the data generated in our laboratory against a number of cancer cell lines. This data clearly demonstrate the potential of compounds of the present invention as anti-cancer agents.

Table 2

Cell Line	Cell Type	^3H Thymidine / MTS* REPRESENTATIVE COMPOUND IC_{50} nM
U2OS	Sarcoma	400
SiHa	Cervical	420
T-24	Bladder	390
A-375	Melanoma	585
Kato III	GIST (gastric)	450
U373MG	Glioblastoma	2000*
A-498	Renal	851*
Mia-Pa-Ca	Pancreatic	1200*

Hep 3B	Liver	2000*
SaSO-2	Osteosarcoma	850*
OVCAR-3	Ovarian	1200*
HT-29	Colon	600
K-562	Chronic Myelogeous leukemia	850
HL-60	Promyelocyctic Leukemia	750
U-937	Histiocytic Lymphoma	350
MOLT-4	Lymphoblastic leukemia	300

Moreover, after five long years of extensive research on Cdk inhibitors presented in this invention, I am of the opinion that these compounds are potential anticancer compounds because of following reasons: (1) induction of cell-cycle arrest in G1 and G2, (2) direct inhibition of Cdk activities by binding to the ATP-binding site, (3) decreasing the level of cyclin D1 followed by loss of Cdk4-D1 enzyme activity and pRb phosphorylation and (4) by induction of apoptosis. The compounds in the present invention can therefore be effective against various solid and haematological malignancies.

11. At page 7 of the office action under the heading "The Amount Of Direction *or Guidance Present and The Presence or Absence Of Working Examples*", the examiner has contended that: "if the activity is to be judged by the toxicity of the compounds to the different cell lines, it seems that the compounds contain no activity towards MDA-MB-231 breast and H-460 lung cell lines. Since the

definition of "not toxic" is less than or equal to 30 % toxic, which would include 0% toxicity."

The toxicity of represented compounds in this invention towards human breast carcinoma MDA-MB-231 and lung carcinoma H-460 cell lines was mentioned as 'non toxic' (i.e. < or = 30 %) only at a concentration of 1 μM of the compounds for a period of 48 hours. That does not imply that the compounds are not cytotoxic because the ^{3}H-Thymidine uptake experiment shows that at the same concentration these show anti-proliferative effect indicating that the cells are not dividing and therefore could be arrested in either G1 or G2/M phase of the cell cycle. Indeed, the toxicity of hydrochloride salt of (+)-*trans*-2-(2-chloro-phenyl)-5,7-dihydroxy-8-(2-hydroxy- methyl-1-methyl-pyrrolidin-3-yl)-chromen-4-one ("the representative compound") increases as the concentration of the compound increases above 1 μM and also increasing the time of exposure. Also, flow cytometric data of the representative compound treated human lung carcinoma cell line H-460 at 1.5 μM up to 72 hours also indicated that the cells undergo apoptosis of 27% to 36% at the end of 48 hours and 72 hours respectively confirming the antiproliferative activity and its ability to induce apoptosis in H-460 cells at concentration 1.5 μM and above. Based on the role of Cdk4 and Cdk1 in cell cycle progression, an inhibitor of these two enzymes would be predicted to produce a G1 or a G2 arrest. We have demonstrated using flow cytometry that representative compound can inhibit tumor cell growth by interrupting cell cycle progression either in G1 or in G2, accompanied by apoptosis. (Please refer to the attached Figure 1)

Thus, the above experiments indicate that when cancer cells are incubated with representative compound for longer period of time and at higher concentrations either three to five IC_{50}s, it induces significantly cells cycle arrest and apoptosis. This effect is more pronounced in fast growing cancer cell lines viz. Human promyelocytic leukemia (HL-60) cell line.

12. At page 7 of the office action under the heading "The Quantity Of Experimentation Needed", the examiner has contended that: "the quantity of

experimentation needed is undue experimentation because one skill in the art would need to determine which cancers can be treated with the compounds of the instant invention, dosages, the method of drug delivery, and any potential combination therapies."

The data provided under the heading "evidence of enablement for the use of compounds of formula 1c in the treatment of various cancers" clearly indicates that no undue experimentation is required and the specification provides adequate direction to persons skill in the art to practise the invention. Moreover, several Cdk inhibitors are currently in clinical trials as a monotherapy as well as combination with currently available cytotoxic and targeted anticancer drugs.

For example, please refer to the publications J Clin Oncol 20, 4074-4082, 2002 and J Clin Oncol 20: 2157-2170, 2002.

13. In summary, based on the teachings in the present application and the level of knowledge in the art, it is my opinion that one skilled in the art could readily understand that all cancers as claimed can be treated by compounds of formula 1c of the present invention.

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that wilful false statements so made are punishable by fine or imprisonment or both, under 18 U.S.C. §1001 and that such wilful statements may jeopardize the validity or enforceability of this application or any patent issued thereon.

Dr. Kalpana S. Joshi Date: September 15, 2006

Figure 1: Representative Compound causes an exclusive G1 arrest of synchronous population of H-460 cells followed by apoptosis from 24 hours onwards, which increases up to 72 hours.